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An Antidiabetic Thiazolidinedione Is a High Affinity Ligand for Peroxisome Proliferator-activated Receptor γ (PPARγ)*

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Thiazolidinedione derivatives are antidiabetic agents that increase the insulin sensitivity of target tissues in animal models of non-insulin-dependent diabetes mellitus. *In vitro*, thiazolidinediones promote adipocyte differentiation of preadipocyte and mesenchymal stem cell lines; however, the molecular basis for this adipogenic effect has remained unclear. Here, we report that thiazolidinediones are potent and selective activators of peroxisome proliferator-activated receptor γ (PPARγ), a member of the nuclear receptor superfamily recently shown to function in adipogenesis. The most potent of these agents, BRL49653, binds to PPARγ with a K_d of approximately 40 nM. Treatment of pluripotent C3H10T1/2 stem cells with BRL49653 results in efficient differentiation to adipocytes. These data are the first demonstration of a high affinity PPAR ligand and provide strong evidence that PPARγ is a molecular target for the adipogenic effects of thiazolidinediones. Furthermore, these data raise the intriguing possibility that PPARγ is a target for the therapeutic actions of this class of compounds.

Adipocytes are highly specialized cells that play a critical role in energy homeostasis. Their primary role is to store triglycerides in times of caloric excess and to mobilize this reserve during periods of nutritional deprivation. Adipocytes are derived from a multipotent stem cell of mesodermal origin that also gives rise to the muscle and cartilage lineages. Studies of the adipocyte differentiation program have been facilitated by the availability of established mesenchymal and preadipocyte cell lines that can be induced to differentiate upon treatment with mixtures of hormonal stimulants (reviewed in Ref. 1).

Adipocyte differentiation is characterized by a coordinate increase in adipocyte-specific gene expression. In most cases, these increases can be accounted for by activation of gene transcription. Thus, considerable effort has been focused on the identification of transcription factors that regulate adipocyte-specific genes. Recently, an orphan member of the nuclear receptor superfamily of ligand-activated transcription factors, designated peroxisome proliferator-activated receptor γ (PPARγ),[1] was shown to be expressed in an adipose-specific manner and its expression induced early during the course of differentiation of several preadipocyte cell lines (2, 3). Subsequent studies revealed that forced expression of PPARγ in fibroblasts resulted in adipocyte differentiation; this differentiation was significantly enhanced in the presence of weak PPARγ activators (4). These data demonstrated that PPARγ plays a pivotal role in the adipogenic signaling cascade.

PPARγ is one of a subfamily of PPARs encoded by independent genes (5). To date, three mammalian PPARs, designated PPARα, PPARγ, and NUC-1, have been identified (6–10). PPARs regulate gene expression by binding to DNA sequence elements termed PPAR response elements. PPAR response elements have been identified in the regulatory regions of a number of genes encoding enzymes that modulate lipid metabolism, indicating a physiological role for the PPAR family in the regulation of lipid homeostasis (reviewed in Ref. 11). PPARs have been shown to be activated to various degrees by micromolar concentrations of long-chain fatty acids and a structurally diverse group of compounds termed peroxisome proliferators that includes the fibrate class of hypolipidemic drugs (5–9, 12). However, as no binding of these compounds to the PPARs has been reported, PPARs have remained "orphan" receptors.

To further examine the role of PPARγ in adipocyte differentiation, we sought to identify activators of PPARγ. We report here that a class of compounds, termed thiazolidinediones (13), are PPARγ-selective ligands. Thiazolidinediones are known to have marked adipogenic effects on preadipocyte and mesenchymal stem cells *in vitro* (14–17) and dramatic antidiabetic effects in animal models of NIDDM (13, 18). Our data provide strong evidence that PPARγ is the molecular target for the adipogenic effects of thiazolidinediones and, furthermore, suggest that PPARγ may be the target for the antidiabetic actions of this class of compounds.

MATERIALS AND METHODS

Plasmids—GAL4-PPAR chimera expression constructs contain the translation initiation sequence and amino acids 1–76 of the glucocorticoid receptor fused to amino acids 1–147 of the yeast transcription factor GAL4, including the DNA binding domain, in the pSG5 expression vector (Stratagene). cDNAs encoding amino acids 167–468, 138–440, and 174–475 of murine PPARα, NUC-1, and PPARγ1 (ref. 8) were amplified by polymerase chain reaction and inserted C-terminal to GAL4 in the pSG5 expression vector (Stratagene) to generate plasmids pSG5-GAL4-PPARα, pSG5-GAL4-NUC-1, and pSG5-GAL4-PPARγ, respectively. The regions of the PPARs included in the chimeras should contain the ligand binding domains based on their homology to ligand binding domains of characterized nuclear receptors (19, 20). The chimeras initially contained the translation start site and N-terminal 262 amino acids of the glucocorticoid receptor, including the τ_1 transcriptional transactivation domain (21). However, as these chimeras had high basal activity in CV-1 cells, a 0.6-kilobase *Bgl*II fragment containing the τ_1 domain was removed, leaving the translation start site and amino acids 1–76 of the glucocorticoid receptor. Wild-type receptor expression vectors were generated by insertion of cDNAs encoding murine PPARα, NUC-1, PPARγ1, and PPARγ2 into the expression

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[1] The abbreviations used are: PPARγ, peroxisome proliferator-activated receptor γ; NIDDM, non-insulin-dependent diabetes mellitus; DME medium, Dulbecco's modified Eagle's medium; CAT, chloramphenicol acetyltransferase; tk, thymidine kinase; GST, glutathione *S*-transferase.

vector pSG5 (Stratagene). Reporter plasmid $(UAS)_5$-tk-CAT was generated by insertion of five copies of a GAL4 DNA binding element into the *Bam*HI site of pBLCAT2 (Ref. 22). The reporter aP2-tk-CAT was generated by insertion of the 518-bp *EcoRI/Xba*I fragment containing the enhancer of the aP2 gene (23) into the *Bam*HI site of pBLCAT2.

Cotransfection Assay—CV-1 cells were plated in 24-well plates in DME medium supplemented with 10% delipidated fetal calf serum. In general, transfection mixes contained 10 ng of receptor expression vector, 100 ng of the reporter plasmid, 200 ng of β-galactosidase expression vector (pCH110, Pharmacia) as internal control, and 200 ng of carrier plasmid. Transfections were done with Lipofectamine (Life Technologies, Inc.) according to the manufacturer's instructions. Cell extracts were prepared and assayed for chloramphenicol acetyltransferase (CAT) and β-galactosidase activities as described previously (24).

Ligand Binding Assay—cDNA encoding amino acids 174–475 of PPARγ1 was amplified via polymerase chain reaction and inserted into bacterial expression vector pGEX-2T (Pharmacia). GST-PPARγ LBD was expressed in BL21(DE3)plysS cells and extracts prepared as described previously (25). For saturation binding analysis, bacterial extracts (100 μg of protein) were incubated at 4 °C for 3 h in buffer containing 10 mM Tris (pH 8.0), 50 mM KCl, 10 mM dithiothreitol with [^{3}H]-BRL49653 (specific activity, 40 Ci/mmol) (18) in the presence or absence of unlabeled BRL49653. Bound was separated from free radioactivity by elution through 1-ml Sephadex G-25 desalting columns (Boehringer Mannheim). Bound radioactivity eluted in the column void volume and was quantitated by liquid scintillation counting.

Adipocyte Differentiation Assay—C3H10T1/2 cells were grown in a 24-well plate in DME medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum. Medium and compound were exchanged every 3 days. Cells were stained at day 7 with Oil Red O and photographed.

Northern Analysis—C3H10T1/2 cells were grown in 225-mm flasks in DME medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum. Medium was exchanged every 3rd day and fresh compound added. Cells were harvested on day 7 and poly(A)$^+$ RNA prepared using the PolyATract® system 1000 (Promega).

RESULTS AND DISCUSSION

Thiazolidinediones are Selective Activators of PPARγ—A transient cotransfection assay was used to screen for PPARγ activators. As mammalian cell lines contain endogenous nuclear receptors that can complicate interpretation of the results, we used an established chimera system (26). Chimeras were constructed that fused the putative ligand binding domains of the three murine PPAR subtypes (α, γ, NUC-1) to the DNA binding domain of the yeast transcription factor GAL4. The structural organization of the GAL4-PPAR chimeras is shown schematically in Fig. 1*A*.

Expression plasmids for the GAL4-PPAR chimeras were cotransfected into CV-1 cells with a reporter construct containing five copies of the GAL4 DNA binding site upstream of the thymidine kinase (tk) promoter driving chloramphenicol acetyltransferase (CAT) gene expression. As previously reported, all three PPAR subtypes were activated by high concentrations of the peroxisome proliferator Wy14,643 (Fig. 1*B*) (5–8, 12). Whereas the PPARα-GAL4 chimera was activated in the presence of 1×10^{-5} M Wy14,643, activation of the PPARγ and NUC-1 chimeras required 1×10^{-4} M Wy14,643 (Fig. 1*B*). Using this assay, we identified four compounds that were efficacious activators of the GAL4-PPARγ chimera at a concentration of 1×10^{-5} M, yet had little or no activity on either the GAL4-PPARα or GAL4-NUC-1 chimeras (Fig. 1*B*), even when concentrations as high as 1×10^{-4} M were used (data not shown). These chemicals failed to activate a control chimera that lacked a ligand binding domain (data not shown). Interestingly, these four compounds, termed BRL49653, pioglitazone, ciglitazone, and englitazone, fall into a class of structurally related antidiabetic agents designated thiazolidinediones (Fig. 1*C*).

The effects of the thiazolidinediones on wild-type PPARγ were examined next. Two isoforms of PPARγ, termed PPARγ1 and PPARγ2, have been identified and shown to differ in their amino termini (2, 8–10). Expression vectors for the two PPARγ isoforms were cotransfected into CV-1 cells with a reporter plasmid containing the enhancer of the adipocyte-specific aP2 gene driving expression of the tk-CAT construct. The aP2 enhancer has previously been shown to contain two PPARγ response elements and to confer responsiveness to pioglitazone (2, 27). Both PPARγ1 and PPARγ2 were activated by BRL49653 and pioglitazone in a dose-dependent and saturable manner (Fig. 2). Interestingly, although the half-maximal concentration of activation with pioglitazone was 4×10^{-7} M for both PPARγ isoforms, the EC_{50} with BRL49653 was 3×10^{-8} M and 1×10^{-7} M for the PPARγ1 and PPARγ2 isoforms, respectively (Fig. 2). Thus, the two PPARγ isoforms have distinct activation profiles. Ciglitazone and englitazone, although less potent, also activated both PPARγ isoforms (Fig. 2). No significant activation of wild-type PPARα or NUC-1 was seen in the presence of 1×10^{-5} M of the thiazolidinediones (data not shown). Likewise, the thiazolidinediones failed to activate other nuclear receptors including the human retinoic acid receptor α and the human thyroid hormone receptor β (data not shown). Thus, the thiazolidinediones are potent and selective

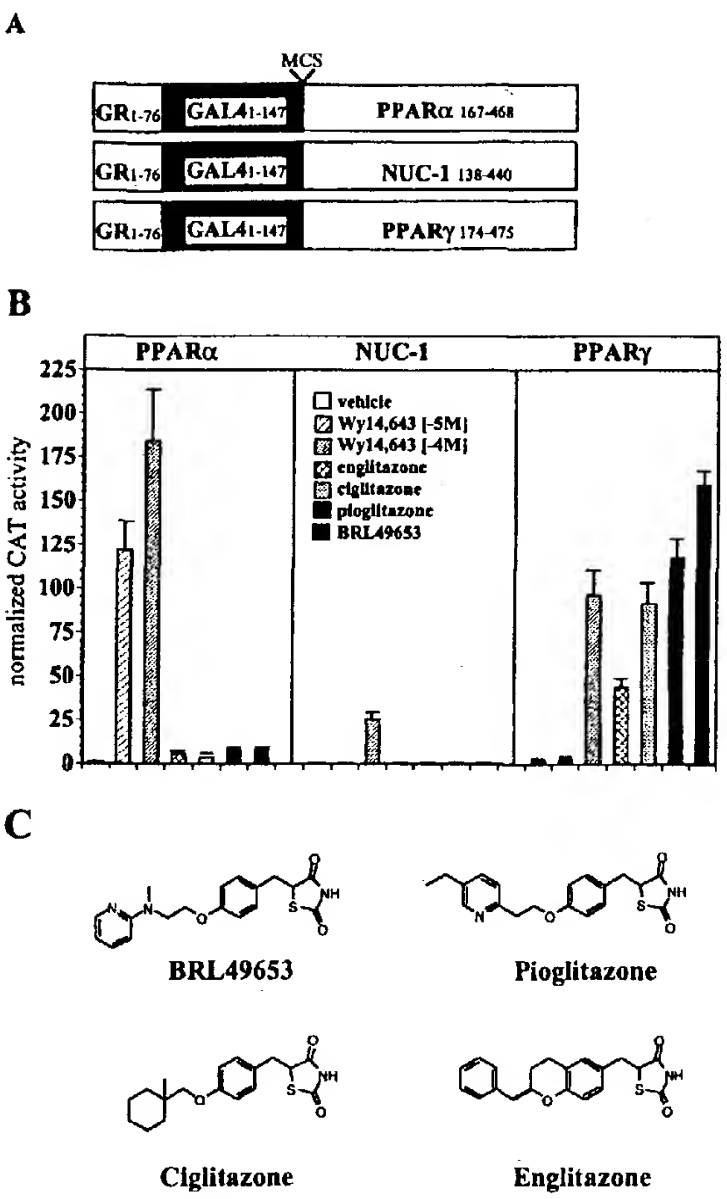

FIG. 1. **Thiazolidinediones are selective activators of PPARγ.** *A*, schematic representation of the chimeras used in this study. Chimeras include the translation initiation site and amino acids 1–76 of the glucocorticoid receptor (*GR*), amino acids 1–147, including the DNA binding domain, of GAL4, and the putative ligand binding domains of PPARα, NUC-1, and PPARγ. See "Materials and Methods" for additional details. *MCS*, multiple cloning site. *B*, CV-1 cells were cotransfected with chimeric receptor expression plasmids pSG5-GAL4-PPARα, pSG5-GAL4-NUC-1, or pSG5-GAL4-PPARγ and the reporter plasmid $(UAS)_5$-tk-CAT. Cells were treated with either vehicle alone (0.1% Me_2SO), 1×10^{-5} M or 1×10^{-4} M Wy14,643, or 1×10^{-5} M thiazolidinediones and cell extracts subsequently assayed for CAT activity. Similar results were obtained in two independent experiments performed in triplicate. *C*, chemical structures of BRL49653, pioglitazone, ciglitazone, and englitazone.

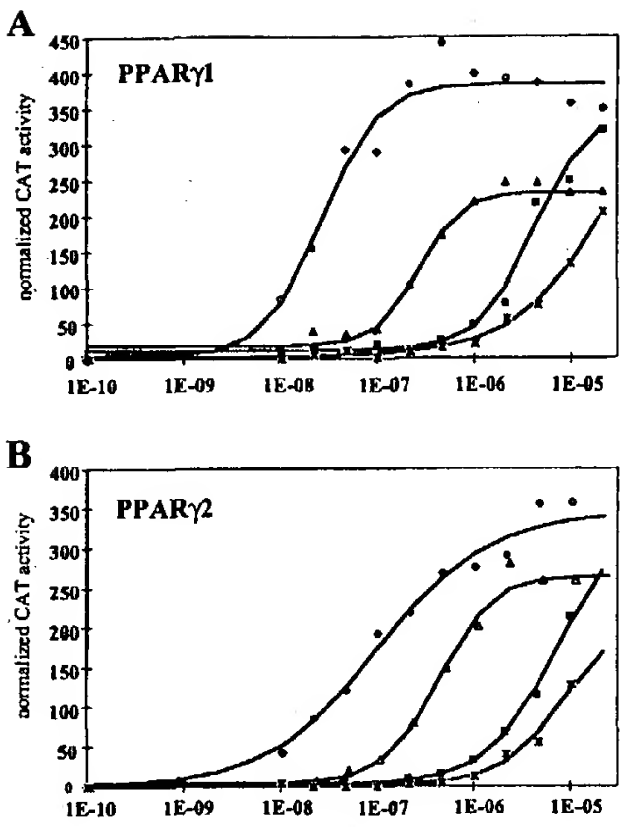

FIG. 2. **Thiazolidinediones activate wild-type PPARγ1 and PPARγ2.** CV-1 cells were cotransfected with expression plasmids for PPARγ1 (*A*) or PPARγ2 (*B*) and reporter plasmid aP2-tk-CAT. Cells were treated with increasing concentrations of BRL49653 (*open diamonds*), pioglitazone (*open triangles*), ciglitazone (*closed squares*), and englitazone (*stars*), and cell extracts subsequently assayed for CAT activity. Similar results were obtained in two independent experiments performed in triplicate.

activators of PPARγ.

Thiazolidinedione BRL49653 Is a High Affinity PPARγ Ligand—Although it has been shown that members of the PPAR subfamily of nuclear receptors are activated by micromolar concentrations of fatty acids and hypolipidemic drugs such as clofibric acid and Wy14,643, none of these compounds have been shown to interact directly with the PPARs (5–9, 12). To test for binding of BRL49653 to PPARγ, the region extending from the end of the conserved DNA binding domain to the C terminus of PPARγ, which includes the putative ligand binding domain (LBD) (19, 20), was expressed in *Escherichia coli* as a fusion protein with glutathione *S*-transferase (GST-PPARγ LBD). Radiolabeled BRL49653 bound specifically and saturably to GST-PPARγ LBD with a K_d of 43 nM (Fig. 3, *A* and *B*). No binding was detected in control extracts from bacteria expressing glutathione *S*-transferase (data not shown). Consistent with the dose-response data for PPARγ in the transient transfection assay, BRL49653 was the most effective competitor for binding of tritiated BRL49653 to GST-PPARγ LBD, followed by pioglitazone (Fig. 3*C*). Ciglitazone and englitazone also competed for binding of tritiated BRL49653 to GST-PPARγ LBD, albeit less efficiently than pioglitazone (Fig. 3*C*). Thus, all four thiazolidinediones bound directly to PPARγ. In control experiments, dexamethasone failed to compete with tritiated BRL49653 for binding to PPARγ (Fig. 3*C*). These data represent the first description of PPAR ligands and demonstrate that PPARγ is a *bona fide* member of the steroid/thyroid hormone/retinoid family of ligand-activated transcription factors.

BRL49653 Promotes Differentiation of C3H10T1/2 Stem Cells to Adipocytes—Induction of PPARγ message is a very early event during the course of differentiation of several preadipocyte cell lines, preceding the induction of other adipocyte markers such as aP2 and C/EBPα (2–4). Recently, it was shown that ectopic expression of PPARγ in fibroblasts induces the entire adipocyte differentiation program as measured by

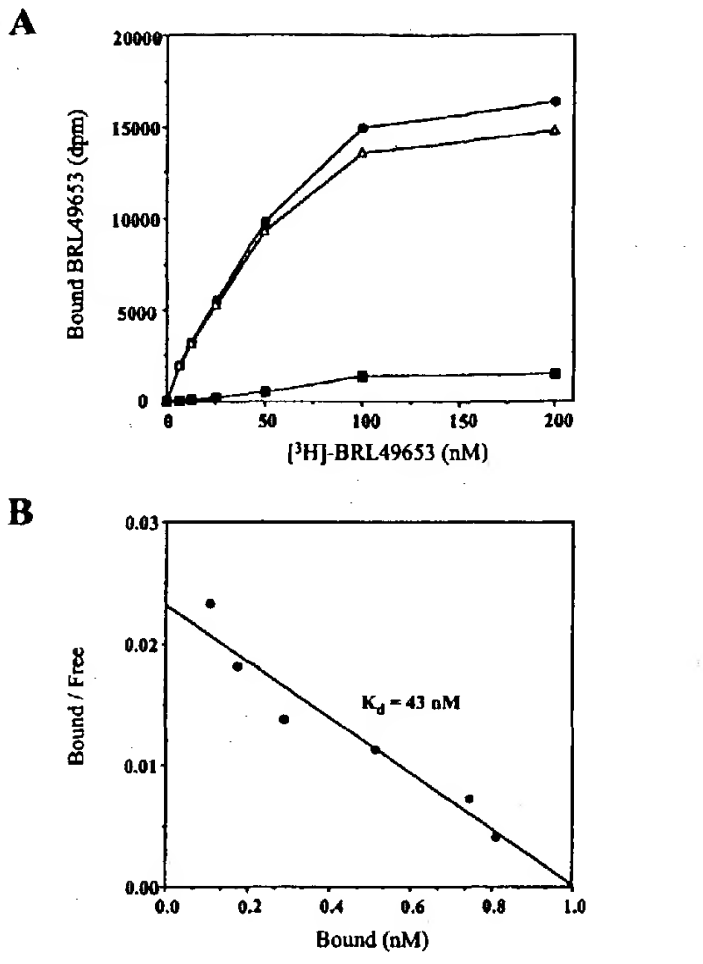

FIG. 3. **The thiazolidinedione BRL49653 binds PPARγ with high affinity.** *A*, bacterial extracts containing the glutathione *S*-transferase-PPARγ ligand binding domain fusion protein (GST-PPARγLBD) were incubated with increasing concentrations of tritiated BRL49643 in the absence (total binding; *closed circles*) or presence (nonspecific binding; *closed squares*) of a 500-fold excess of nontritiated BRL49653. Specific binding of tritiated BRL49653 to GST-PPARγLBD is indicated (*open triangles*). *B*, Scatchard analysis. Specific binding of BRL49653 in *A* was transformed by Scatchard analysis and plotted. Linear regression yielded a K_d of 43 nM. Similar results were obtained in three independent experiments performed in duplicate. *C*, competition binding analysis was performed with GST-PPARγLBD and 10 nM tritiated BRL49653 in the presence of a 100-, 500-, or 2500-fold excess of unlabeled BRL49653 (*closed squares*), pioglitazone (*open squares*), englitazone (*closed circles*), ciglitazone (*open circles*), or dexamethasone (*closed triangles*). Similar results were obtained in two independent experiments performed in duplicate.

lipid accumulation and induction of adipocyte-specific genes (4). These data demonstrate a causal role for PPARγ in adipogenesis. To test for an effect of a PPARγ-selective ligand on adipogenesis, we treated pluripotent C3H10T1/2 stem cells with BRL49653. Untreated C3H10T1/2 cells express PPARγ as demonstrated by Northern analysis (Fig. 4*C*). Treatment of C3H10T1/2 cells with BRL49653 resulted in efficient adipocyte differentiation as judged by Oil Red O staining (Fig. 4, *A* and *B*)

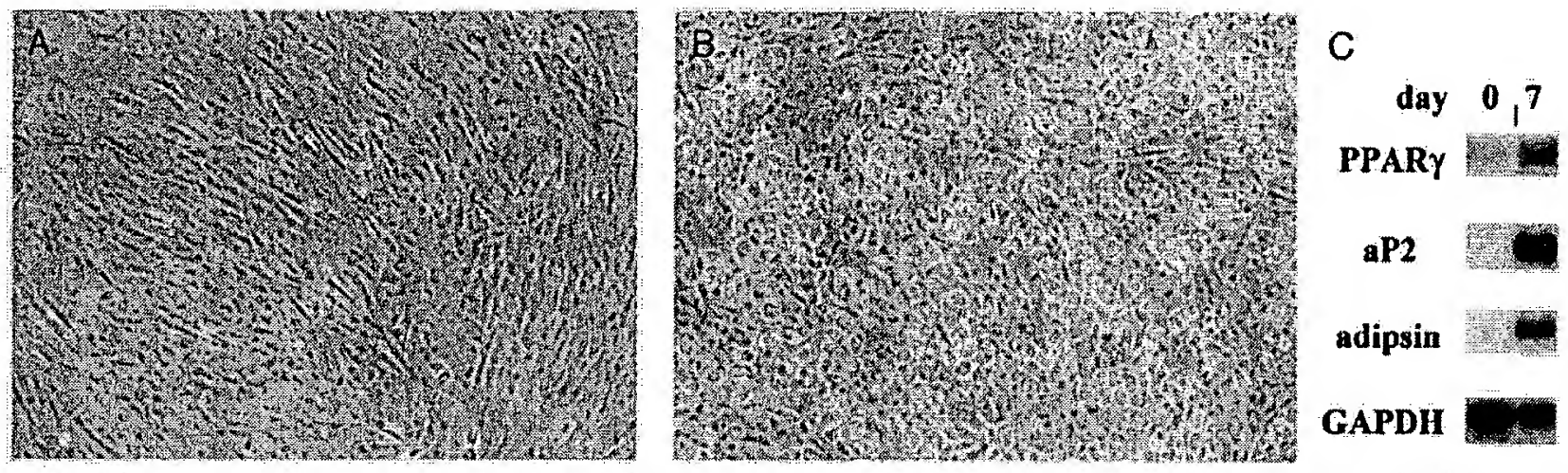

FIG. 4. **BRL49653 promotes differentiation of C3H10T1/2 cells to adipocytes.** C3H10T1/2 cells were treated for 7 days with either vehicle alone (0.1% Me_2SO) (*A*) or 1×10^{-6} M BRL49653 (*B*) and subsequently stained for lipid accumulation with Oil Red O. Magnification is × 40. *C*, Northern analysis was performed with 2 μg of poly(A)$^+$ RNA prepared from C3H10T1/2 cells that were either untreated (day 0) or treated for 7 days with 1×10^{-5} M BRL49653. Blots were hybridized with ^{32}P-labeled aP2, adipsin, PPARγ, and GAPDH cDNA probes. An equivalent amount of intact RNA was run in each lane as indicated by the GAPDH cDNA probe. Exposure times were 1.5, 24, 48, and 7 h for aP2, adipsin, PPARγ, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) blots, respectively.

and Northern analysis using the adipocyte-specific markers aP2 and adipsin (Fig. 4*C*). PPARγ expression levels were increased approximately 3-fold in response to treatment with BRL49653 (Fig. 4*C*). Significant adipocyte differentiation was seen at concentrations of BRL49653 as low as 1×10^{-7} M (data not shown). These data provide evidence that ligand-mediated activation of PPARγ is sufficient to initiate the adipogenic signaling cascade in a mesenchymal stem cell line. Furthermore, our results provide a mechanistic explanation for the reported adipogenic effects of several thiazolidinediones on preadipocyte cell lines (14–17).

Thiazolidinediones are insulin sensitizers that significantly reduce glucose, lipid, and insulin levels in animal models of NIDDM and obesity (13). Our results suggest that PPARγ may be a target for the antidiabetic effects of these agents. How might activation of an adipocyte-specific transcription factor account for these diverse therapeutic effects? In recent years there has been a growing awareness that NIDDM is not only a derangement of glucose homeostasis, but is also characterized by elevated levels of circulating lipids. Although the mechanism is poorly characterized, increases in lipid levels have been shown to interfere with glucose disposal (reviewed in Ref. 28). Thus, activation of PPARγ in adipose may affect glucose usage in other tissues such as skeletal muscle, the primary site of glucose disposal, through an indirect mechanism involving modulation of lipid levels. Alternatively, activation of PPARγ may regulate signaling molecules secreted by adipose such as tumor necrosis factor-α or the *ob* gene product (29, 30). These secreted products could, in turn, modulate glucose metabolism in other tissues.

In summary, our demonstration that an adipogenic thiazolidinedione is a high affinity ligand for PPARγ provides compelling evidence that this nuclear receptor plays a critical role in adipogenesis. Clearly, an understanding of the PPARγ signaling cascade may lead to insights into the molecular mechanisms regulating energy homeostasis and the defects underlying obesity and NIDDM.

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